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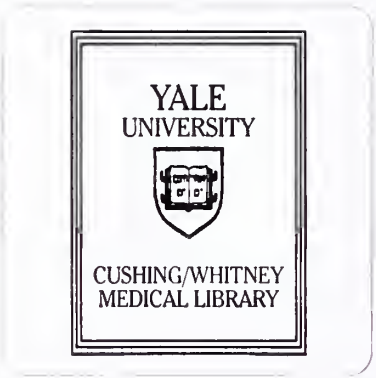
MODULATION OF ATP-SENSITIVE POTASSIUM CHANNEL ACTIVITY BY ATP-BINDING CASSETTE TRANSPORTERS: A MODEL FOR CHANNEL REGULATION

Tong-Yi Yao

Yale University

1998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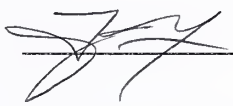

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MODULATION OF ATP-SENSITIVE POTASSIUM CHANNEL ACTIVITY BY ATP-BINDING CASSETTE TRANSPORTERS: A MODEL FOR CHANNEL REGULATION

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Tong-Yi Yao

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MODULATION OF ATP-SENSITIVE POTASSIUM CHANNEL ACTIVITY BY ATP-BINDING CASSETTE TRANSPORTERS: A MODEL FOR CHANNEL REGULATION

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The aim of this study was to characterize the relationship between ATP-Binding Cassette (ABC) transporters and heterologous ion channels. Our hypothesis was that regulation of ATP-sensitive potassium channel activity by ABC transporters is through direct interactions dependent upon motifs specific to this superfamily. If the hypothesis is valid, it suggests that certain ABC transporters may be able to interact with numerous ion channels. Furthermore, if direct coupling of specific domains or residues on the ABC transporter and channel protein are necessary, mutations in either protein would disrupt this interaction, diminishing the ABC transporters ability to modulate channel activity.

Using two-electrode voltage clamping, this study examined two ABC transporters, the cystic fibrosis transmembrane conductance regulator (CFTR) and the pancreatic beta cell high-affinity sulfonylurea receptor (SUR1), and their interactions with "associated" inwardly-rectifying ATP-sensitive potassium channels, the renal potassium channels (ROMK1/Kir1.1a and ROMK2/Kir1.1b) and the pancreatic potassium channel (BIR/Kir6.2). Expression of either ROMK1 (Kir1.1a) or ROMK2 (Kir1.1b) alone in Xenopus oocytes resulted in barium-sensitive potassium currents with minimal to moderate sulfonylurea (glibenclamide) sensitivity. Coexpression of either CFTR or SUR1 with ROMK2 (Kir1.1b) greatly enhanced the sensitivity of the potassium currents to sulfonylurea. In contrast, neither the expression of CFTR or SUR1 with ROMK1 (Kir1.1a) enhanced the effects of glibenclamide on potassium currents. In fact, the expression of CFTR with ROMK1 (Kir1.1a) decreased the glibenclamide effect. These data suggest that the interaction of ABC transporters with ion channels is dependent upon characteristics of both subunits.

Lastly, we examined the clinical impact of disrupting the interactions between ABC transporters and ATP-sensitive potassium channels in a case of familial hyperinsulinemic hypoglycemia of infancy (PHHI). In accordance with our above findings, coexpression of SUR1 with a BIR (Kir6.2) mutant

identified in an infant with PHHI failed to produce a potassium current, demonstrating that a nonfunctional BIR (Kir6.2) mutant resulted in a potassium channel that was nonfunctional.

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As with so many other accomplishments in my life, I was sustained throughout this process by my family.

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1. Introduction

1-1. ATP-Binding Cassette Transporter Superfamily

1-1.1. The Typical ABC Transporter

The ATP-binding cassette (ABC) transporters are a superfamily of over 100 transmembrane proteins characterized by a highly conserved ATP-binding cassette, or nucleotide binding fold (NBF) (Higgins, 1992; Higgins, 1995). In general, their structure consists of four core domains—two transmembrane domains and two nucleotide-binding domains (Figure 1). In prokaryotes, the four domains exist more commonly as separate polypeptides while in eukaryotes they tend to exist as fused multidomain complexes. Whether expressed as separate polypeptides or as a multidomain protein, the transmembrane domains appear to associate with and anchor the two nucleotide-binding domains. Several bacterial transporters appear to have a highly conserved short sequence on a cytoplasmic loop of the transmembrane domain which may interact with the nucleotide-binding domains. Members of this superfamily function as selective active transporters or channels. ABC transporters may either export or import substrate; none, at present, demonstrate bidirectional transport. Substrates range from simple inorganic ions, sugars, and amino acids to more complex polysaccharides and proteins.

While the ABC transporter superfamily is defined by the basic four domain complex described above, there is limited sequence conservation between different proteins in the family. The highest degree of sequence identity is found in the nucleotide-binding domains. No experimental data (NMR, crystallographic, microscopic) detailing the exact structure of ABC transporters is presently available. Computer-generated predictions based upon the amino acid sequence suggest that each transmembrane domain consists of multiple alpha-helical membrane-spanning segments with the N- and C- termini are usually present on the cytoplasmic side. Exceptions such as the sulfonylurea receptor whose N-terminus is extracellular do exist. Although a typical ABC transporter has six segments, significant

variation in the number of alpha-helical membrane-spanning segments present in each transmembrane domain occurs.

1-1.1.1. Role of the Transmembrane Domains

The transmembrane domains are believed to form the path for substrate transport and determine substrate specificity (Higgins, 1992; Higgins, 1995). As the proteins are involved in the transport of highly diverse substrates and as the amino acid residues responsible for specificity need not be adjacent on the primary sequence, the amino acid sequences of the transmembrane domains exhibit great variability when comparing between different proteins. However, sequence similarities that do exist between transporters do not necessarily translate into similarities in substrate specificity. When the two transmembrane domains present in each individual ABC transporter are compared there is generally greater sequence identity present. In ABC transporters with only a single transmembrane domain, it may function as a homodimer. Greater sequence identity is found between the two distinct transmembrane domains of a single ABC transporter suggesting they may function together as a pseudodimer.

1-1.1.2. Role of the Nucleotide-Binding Domains

The highly hydrophilic nucleotide-binding domains reside on the cytosolic side of the membrane where their nucleotide binding folds interact with cytosolic nucleotides (Higgins, 1992; Higgins, 1995). These domains typically couple the energy of ATP hydrolysis to the transport of substrates across the membrane perhaps through conformational changes affecting the transmembrane domains. There is a high degree of sequence conservation in these domains between ABC transporters with up to 30-50% sequence identity depending on the proteins compared. Greater sequence identity is generally present between the two nucleotide-binding domains of a single transporter. This sequence identity includes the Walker A and Walker B motifs which are responsible for

interacting with the phosphates of nucleotides and associated with many other nucleotide-binding proteins. The presence of both nucleotide-binding domains is necessary for the function of the protein; however, the two nucleotide-binding domains are not necessarily functionally equivalent. For example, in P-glycoprotein, mutations in either domain impacts adversely on transport activity. However, in cystic fibrosis many mutations in the first (or N-terminal) nucleotide-binding domain of CFTR result in a disease state but a mutation in the C-terminal nucleotide-binding domain does not appear to inhibit CFTR function (Welsh and Smith, 1993).

1-1.2. ABC Transporters and Disease

ABC transporters are now known to be involved in a variety of human diseases (Table 1) ranging from cystic fibrosis (CF) and persistent hyperinsulinemic hypoglycemia of infancy (PHHI), which are our focus in these studies, to other recessively inherited diseases such as X-linked adrenoleukodystrophy, Zellweger syndrome, and Stargardt macular dystrophy as well as multifactorial diseases such as noninsulin-dependent diabetes. In addition, ABC transporters affect the course of human disease by conferring chemotherapeutic drug resistance to cancer cells as well as antibiotic resistance to a variety of infectious microorganisms.

1-1.3. ABC Transporters as Channel Regulators

Studies have suggested that ABC transporters may interact with heterologous ion channel proteins regulating their activity (Higgins, 1995). This regulatory function may be as an extension of their intrinsic transporter or channel functions or a result of either direct or indirect interactions with these other channel proteins. Understanding how they function as channel regulators may give insight into the pathophysiology of the diseases associated with ABC transporter dysfunction and may provide new targets for treatment of the disease process.

1-1.3.1. Mechanisms of Channel Regulation

Four major models of regulation above the level of gene expression have been proposed to explain the mechanism by which ABC transporters may regulate other channel proteins (Figure 2) (Higgins, 1995). The first model assumes that a substrate (X) transported by the ABC protein acts as regulatory molecule to modulate channel activity in an autocrine fashion. In this model, the ABC protein's regulation of another channel is a consequence of its intrinsic transporter function. The second model proposes direct interactions between the ABC transporter and the channel protein as the basis of modulation of channel activity. These could involve protein-protein interactions or the formation of an ABC transporter-channel protein complex. The third and fourth models are of indirect interactions not associated with the ABC protein's transporter function; one looks upon the ABC transporter as a facilitator of increased insertion of channel proteins into the membrane while the other model postulates the coupling of the ABC transporter with intermediate protein molecules, possibly cytoskeletal components, thereby effecting the channel protein.

Our studies focus upon the direct model of channel regulation. Direct interactions appear to explain how CFTR and SUR1 function as channel regulators. More specifically, the regulation of channel activity appears to be dependent upon the presence of specific motifs of ABC transporters, such as the NBFs (McNicholas et al., 1997).

1-1.3.2. Multidrug Resistance Gene Product P-glycoprotein

P-glycoprotein is responsible for the multidrug resistance of cancers to chemotherapeutic drugs. P-gp functions as an active exporter of hydrophobic compounds including chemotherapeutic drugs. When overexpressed in cancer cells P-gp confers upon them multidrug resistance by reducing the cytoplasmic concentrations and cytotoxicity of the chemotherapeutic drugs. The energy to pump the drugs out of the cell is derived from

ATP hydrolysis. P-gp expression is also associated with increased volume-activated Cl^- channel currents suggesting that it may be a multifunctional protein with an additional physiological function as a regulator of epithelial cell volume (Gill et al., 1992; Valverde et al., 1992). The volume-activated Cl^- channel appears to be sensitive to osmotic gradients. P-gp appears to affect the channel activation through altering this sensitivity as well as imposing a dependency on protein kinase C.

1-1.4. Promiscuity of ABC Transporters

Studies have suggested that ABC transporters may be able to substitute for other members of the family in their interactions with heterologous proteins (Ammala et al., 1996). Whether this ability to substitute for other members of the family is a consequence of similarities in motifs allowing for equivalent direct coupling of ABC transporters with channel proteins is unclear. Another possibility is that ABC transporters capable of exporting or importing similar substrates may substitute for one another. For example, such a relationship was found to exist between two ABC transporters, P-gp and STE6 which is responsible for exporting a-factor mating peptide in yeast (Raymond et al., 1992). When the mouse *mdr3* gene which produces P-pg was expressed in a yeast cell with an *ste6* deletion, the cell regained its ability to export a-factor. In this study, a mutation known to affect the activity of P-gp was then introduced into the *mdr3* gene. Coexpression of this mutant with the STE6 deficient yeast cell did not restore function. Studies done coexpressing SUR1 with inwardly rectifying potassium channels ($K_{ir}1.1a$ and $K_{ir}6.1$) other than BIR (Kir6.2) suggest that it is able to interact with multiple K_{ir} channels and confer sulfonylurea sensitivity (Ammala et al., 1996).

1-2. Cystic Fibrosis Transmembrane Conductance Regulator

The cystic fibrosis transmembrane conductance regulator (CFTR) is a somewhat atypical ABC transporter. Unlike the vast majority of ABC transporters, CFTR functions

as a chloride channel not as a transporter and possesses a five domain structure—two transmembrane domains, two nucleotide-binding domains, and an additional regulatory R-domain which appears to block the channel pore (Figure 3) (Fuller and Benos, 1992; Riordan, 1993; Tsui, 1995; Davis et al., 1996). Phosphorylation of this R-domain by a cyclic AMP-dependent protein kinase A (PKA) is required for channel activation. Maintenance of channel activity requires the continued presence of ATP at the NBFs. In addition, there appear to be other phosphorylation sites which may modulate channel kinetics and activity as well.

1-2.1. Role in Regulation of Respiratory Secretions

Normal function of CFTR appears important for effective mucociliary clearance, the mechanism by which inhaled foreign matter are removed from the lungs. Respiratory secretions are critical in the maintenance of mucociliary clearance (Welsh, 1988). The two components of the system are the cilia of the epithelial cells and the respiratory fluids. The respiratory fluids can be conceived as a bilayer with a discontinuous mucus layer, or gel phase, overlying a periciliary fluid layer, or sol phase, in which the cilia are immersed. The mucus layer serves to trap inhaled particles whereas the periciliary fluid provides a watery medium in which the cilia can beat freely. The tips of the cilia are in contact with the mucus facilitating its movement along with the foreign matter towards the larynx.

A number of features of the airway epithelium are critical for the maintenance of electrolyte and water transport necessary to ensure the appropriate quantity and composition of the periciliary fluid as well as the quality of the mucus. The airway epithelial cells are polar with distinct apical and basolateral surfaces. The ciliated apical membrane facing the lumen and the basolateral membrane facing the interstitial space differ in the types of receptors, transporters, and channels present. The two cell surfaces are further differentiated by the presence of tight junctions joining the epithelial cells along their apical surfaces producing a continuous barrier between the luminal and interstitial spaces. This

barrier serves as a semipermeable membrane allowing regulated solute and water movement with net secretion or absorption. Two pathways of movement across the airway epithelium exist—a paracellular pathway between cells at the tight junctions and a cellular pathway through the epithelial cells themselves.

The interplay between chloride secretion and sodium absorption determines whether fluid is secreted or absorbed by the epithelium, respectively (Welsh, 1988). Whether one or the other process predominates is dependent upon the neurohumoral environment and the anatomical region of the airway. Both, however, are dependent upon cellular and paracellular pathways. Chloride secretion "begins" with chloride entry across the basolateral membrane coupled to sodium entry. This is an electrically neutral cotransport process in which chloride moves against its electrochemical gradient while sodium moves down its electrochemical gradient. Whether the cotransport process involves a Na^+/Cl^- cotransporter or a $Na^+/K^+/2Cl^-$ cotransporter is dependent upon cell type. The chloride then moves passively across the apical membrane down it electrochemical gradient through chloride channels. Some of the apical chloride channels involved are the cystic fibrosis transmembrane regulator (CFTR) chloride channel, the outwardly rectifying chloride channel (ORCC), and the calcium-activated chloride channel. The basolateral Na^+/K^+ pump, or Na^+,K^+-ATPase, is responsible for maintaining the low intracellular sodium concentrations and negative intracellular voltage necessary for this chloride secretion. The intracellular potassium accumulated through the Na^+,K^+-ATPase as well as the $Na^+/K^+/2Cl^-$ cotransporter passively leaves the cell through a basolateral potassium channel. This basolateral potassium channel also serves to maintain a negative intracellular voltage conductive to chloride leaving the cell at the apical membrane. The net result of the depolarizing transport of chloride across the apical membrane and the hyperpolarizing transport of potassium across the basolateral membrane is a transepithelial voltage which drives the paracellular flow of sodium from the interstitial space to the lumen (Figure 4).

Sodium absorption "begins" with sodium entry across the apical membrane through sodium channels down its electrochemical gradient. The Na^+,K^+-ATPase then pumps sodium against its electrochemical gradient across the basolateral membrane.

This balance of secretion and absorption impacts upon mucociliary clearance through two mechanisms—the hydration of the mucus layer and the depth of the periciliary fluid layer. Airway mucus is produced and secreted primarily by the submucosal glands; however, additional secretory products of goblet cells, ciliated cells, and Clara cells also contribute to its composition. As fluid secretion decreases when chloride secretion decreases or sodium absorption increases, the mucus and periciliary layers become dehydrated making the mucus more viscous and the periciliary layer too shallow for effective ciliary beating. Likewise, fluid result in a periciliary layer which is too deep preventing the cilia tips from contacting with and moving the mucus layer.

1-2.2. Role in Cystic Fibrosis

Cystic Fibrosis (CF) is a common genetic disease affecting the Caucasian population with a frequency of approximately one in 2500 live births. This autosomal recessive disease affects multiple organ systems including the respiratory, gastrointestinal, and reproductive systems. The underlying defect in CF is in the gene encoding CFTR located on chromosome 7 (Riordan et al., 1989; Rommens et al., 1989). Although the gene was not identified until 1989 by Riordan et al., studies by Quinton and Knowles and Boucher during the early 1980s had already demonstrated the primary defect as a decrease in chloride permeability. The characteristic clinical findings of mucus plugging and infection in the airways, pancreatic insufficiency, and elevated sweat chloride concentrations are a result of this alteration of chloride permeability in secretory epithelial cells in the airways, exocrine pancreas, and sweat glands. Recently, Smith et al. postulated that this alteration in chloride and sodium permeability may lead to changes in airway surface fluid which, in turn, may alter the natural defenses of the lung by inactivating salt-

sensitive peptides, such as defensins (Smith et al., 1996). Currently, the progressive airway disease—decreased mucociliary clearance, mucus plugging, bronchiectasis, and ultimately respiratory failure—is the major cause of morbidity and mortality in CF patients (Fuller and Benos, 1992; Riordan, 1993; Tsui, 1995; Davis et al., 1996).

Mutations resulting in the loss or reduction of CFTR's chloride conductance can be classified into four categories dependent on where they impact on the "pathway" of CFTR production and function following insertion into the cell membrane (Welsh and Smith, 1993). This "pathway" can be conceptually divided into four areas: protein production, protein processing/trafficking, channel regulation/activation, and chloride conduction (Table 2). Defects in channel regulation/activation can also be conceptualized as defects within the nucleotide binding domains, specifically the NBF, as they are intimately involved in channel regulation/activation. Likewise, defects in chloride conduction appear to be localized to the transmembrane domains which are postulated to form the channel pore. The most common mutation is a single amino acid deletion of phenylalanine at position 508 (ΔF508). The ΔF508 mutation results in defective protein processing/trafficking. The ΔF508 protein appears to be incorrectly folded and therefore not trafficked to the Golgi apparatus where it would otherwise be fully glycosylated and subsequently transported to the cell membrane for insertion.

1-2.3. CFTR as Regulator of Heterologous Channel Activity

CF-affected cells exhibit a number of ion transport abnormalities—increased sodium absorption, abnormal regulation of outwardly rectifying chloride channels (ORCCs), and increased calcium-activated chloride channel activity—which can not be explained solely by CFTR's function as a chloride channel. CFTR appears to be a cAMP-dependent regulator of amiloride-sensitive epithelial sodium channels (ENaC) (Stutts et al., 1995; Mall et al., 1996). The mechanism of this interaction while presumed to be direct, however, is unclear. CFTR regulation of ORCCs, which are present in the apical

membranes of airway epithelium, may involve the release of ATP to the extracellular surface (Gabriel et al., 1993; Jovov et al., 1995; Schwiebert et al., 1995). Whether the ATP is transported by CFTR, by an associated channel regulated by CFTR, or by another mechanism is unclear. What is evident is that in CF-affected cells the normal regulation of ORCCs by PKA and ATP is defective and that expression of wild type CFTR in these same cells corrects the defect (Egan et al., 1992). The increase in calcium-activated chloride channel activity appears to be a result of up-regulation (Grubb et al., 1994). Correction of the calcium-activated chloride channel activity and ENaC activity when CF-affected cells are transfected with CFTR supports the notion that CFTR is critical to the regulation of these channels.

1-2.3.1. CFTR and Potassium Channels

There is also evidence that CFTR can modify K_{ATP} channel activity. The ROMK family of apically-located renal K_{ATP} channels is a subgroup of the inwardly rectifying potassium (K_{ir}) channel superfamily (Hebert and Ko, 1994; Zhou et al., 1994; Boim et al., 1995). K_{ir} channels maintain the membrane potential (E_m) of numerous excitable and non-excitable cells near the potassium reversal potential (E_K) (Fakler and Ruppersburg, 1996; Ackerman and Clapham, 1997). K_{ATP} channels within the apical membrane of distal nephron segments of the kidney play a major role in potassium homeostasis. Multiple isoforms of ROMK have been identified. ROMK2 (Kir1.1b), an alternate splice isoform with 19 fewer amino acid residues present on the N-terminus than ROMK1 (Kir1.1a), maintains many of the characteristics of native renal K_{ATP} channels; however, it lacks the same sensitivity to sulfonylureas such as glibenclamide. Coexpression of CFTR, which is present in the nephron, with ROMK2 (Kir1.1b) appears to confer this glibenclamide sensitivity to the K_{ATP} channel (McNicholas et al., 1996). As this effect was not found to be dependent upon PKA and ATP, active transport by CFTR does not appear to be necessary, suggesting a direct interaction between CFTR and ROMK. A recent study

demonstrated CFTR as localizing to the apical membrane of the cortical collecting duct where ROMK2 (Kir1.1b) has previously been found (Todd-tura et al., 1996). In light of these studies, a role for CFTR in the regulation of ROMK channel activity appears possible.

This ability of CFTR to regulate K_{ATP} channel activity is also present in another member of the ABC transporter superfamily, SUR1.

1-3. Sulfonylurea Receptor

The complementary DNA for SUR1 was first cloned in 1995 by Aguilar-Bryan et al. classifying it as a member of the ABC transporter family because it showed significant sequence similarities to other members of the superfamily such as P-gp and CFTR (Aguilar-Bryan et al., 1995). Two nucleotide binding folds with Walker A and B consensus sequences were identified. SUR1, however, has unique structural characteristics. Although a multidomain protein like many other eukaryotic ABC transporters, it possesses nine and four segment transmembrane domains alternating with the two nucleotide-binding domains. Its N-terminus is also located extracellularly. SUR1's role in pancreatic beta cell K_{ATP} channel activity was initially unclear because, as a member of the ABC transporter superfamily, it could function either as the potassium transporter itself or as the regulator of a separate potassium channel subunit. Expression of SUR1 alone resulted in no channel activity, suggesting that coupling with BIR (Kir6.2) was necessary for a functional K_{ATP} channel.

1-3.1. Role in Regulation of Glucose Homeostasis

The pancreatic beta cell ATP-sensitive potassium (K_{ATP}) channel is postulated to be a complex of two subunits, SUR1 and BIR (Kir6.2) (Figure 5) (Philipson and Steiner, 1995; Inagaki et al., 1995). SUR1 appears to serve as the regulator of channel activity while BIR (Kir6.2) forms the channel pore. Studies in which expression of either SUR1

or BIR (Kir6.2) alone did not yield K^+ currents above the endogenous levels of the cell system support the theory that they act as a functional unit (Aguilar-Bryan and Bryan, 1996; Gribble et al., 1997). SUR1 was originally of clinical interest because of the use of sulfonylureas as oral hypoglycemics to stimulate insulin secretion from pancreatic beta cells in the treatment of non-insulin dependent diabetes mellitus (NIDDM).

In physiologic regulation of glucose homeostasis, the K_{ATP} current (I_{KATP}) sets the resting membrane potential for the pancreatic beta cell (Aguilar-Bryan and Bryan, 1996). Increases in the glucose concentration translate into an increase in the cytosolic ATP to ADP ratio which closes the K_{ATP} channel and blocks the outflow of potassium. The subsequent depolarization of the membrane results in the activation of voltage-dependent L-type calcium channels. The calcium influx triggers exocytosis and insulin release (Figure 6). Sulfonylureas such as glibenclamide bring about insulin release through inhibition of the K_{ATP} channel.

1-3.2. Role in Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI)

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) is a rare autosomal recessive disorder of glucose metabolism characterized by recurrent episodes of profound hypoglycemia associated with abnormal regulation of insulin secretion (Aguilar-Bryan and Bryan, 1996; Permutt et al., 1996). These episodes of severe hypoglycemia can present in the early newborn period or as late as several months of age. In all cases, whether the plasma insulin levels are high normal or grossly elevated, there is inappropriately high levels of insulin during hypoglycemia. In order to avoid hypoglycemia, patients require parenteral glucose, often in high doses. Current treatment strategies include pharmacologic interventions such as diazoxide or a somatostatin analog, Octreotide, as well as surgical options such as partial pancreatectomy for refractory hyperinsulinism. Although the disease was described as early as 1953 by McQuarrie and has also been referred to as familial hyperinsulinism and pancreatic nesidioblastosis, the molecular basis was not

elucidated until 1995 when Aguilar-Bryan et al. cloned the gene and Thomas et al. reported the first findings of mutations in SUR1 in families with PHHI (Aguilar-Bryan et al., 1995; Thomas et al., 1995). PHHI has an incidence of one in 50,000 live births in the western European population. In populations such as the Saudi Arabians where there is a high percentage of consanguineous matings, the incidence is much higher, approximately one in 2500 live births. The existence of such families with PHHI facilitated mapping the gene to chromosome 11p15.1 by linkage analysis. Both SUR1 and BIR (Kir6.2) are clustered on chromosome 11p15.1 with the BIR (Kir6.2) gene immediately 3' of the SUR1 gene. Recent studies have attributed patient cases to mutations in the first and second nucleotide-binding fold regions of SUR1 as well as to mutations in BIR (Kir6.2) (Thomas et al., 1996). In some cases these mutations led to truncated proteins while in others they disrupted RNA processing. Both nucleotide-binding domains, however, appear to be necessary for normal channel activity. It appears that mutations in either SUR1 or BIR (Kir6.2) could result in continuous membrane depolarization and subsequent continuous secretion of insulin.

Mutations in SUR1 have also been implicated in NIDDM suggesting that the BIR (Kir6.2)-SUR1 complex plays a pivotal role in the regulation of insulin secretion and maintenance of glucose homeostasis (Nestorowicz, et al., 1996).

1-3.3. SUR1 as Regulator of Heterologous Channel Activity

SUR1 has been demonstrated to interact with numerous K_{ATP} channels other than BIR (Kir6.2), affecting their regulation by sulfonylureas (Ammala et al., 1996). These studies have suggested that interactions between ABC transporters and certain ion channels may be promiscuous.

2. Statement of Purpose

Our hypothesis is that ABC transporters are multifunctional proteins that act not only as active transporters or channels, but also as regulators of heterologous channels. More specifically, we hypothesize that ABC transporters regulate heterologous channel activity via direct interactions dependent upon motifs specific to ABC transporters. If direct coupling of specific domains or residues on the ABC transporter and channel protein is necessary, mutations in either protein would disrupt this interaction diminishing the ABC transporters ability to modulate channel activity. To examine the possibility of ABC transporters substituting for one another in their interactions with heterologous channel proteins, studies were done looking at possible relationships between ABC transporters (CFTR and SUR1) and K_{ATP} channels (ROMK1/Kir1.1a, ROMK2/Kir1.1b, and BIR/Kir6.2). In addition, preliminary studies examining the interactions between SUR1 (wild type) and BIR/Kir6.2 (wild type and mutant) were performed. Together, these experiments will help determine the structure-function relationship responsible for the interaction between ABC transporters and channel proteins and elucidate what regions of the ABC transporters are necessary for channel regulation.

All two-electrode voltage clamp studies were performed upon mRNA-injected Xenopus oocytes. More specifically, the following questions were addressed in this study:

1) Do mutations in specific domains of CFTR impair its ability to interact with the channel protein ROMK2 (Kir1.1b) thus altering the channel activity or glibenclamide sensitivity of the coexpressed peptides?
2) Can SUR1 substitute for CFTR in the CFTR/ROMK interaction?
3) Do alterations in ROMK affect its ability to interact with CFTR thus altering the channel activity or glibenclamide sensitivity of the coexpressed peptides?

4) Do mutations in specific domains of either SUR1 or BIR (Kir6.2) impair their ability to interact thus altering the glibenclamide sensitivity of the coexpressed peptides?

3. Methods

3-1. Xenopus Oocyte Expression System

The Xenopus laevis oocyte expression system was first introduced in 1971 by Gurdon as a means to study the control of gene expression (Gurdon et al., 1971). In 1982, Miledi et al. demonstrated that injection of exogenous mRNA into the cytoplasm resulted in expression of ion channels and receptors. As stage V and VI oocytes possess adequate protein translation and modification machinery, it has proven to be a reliable expression system for the study of ion channels and transporters (Sherman-Gold, 1993). The oocytes are readily harvested through partial ovariectomy through a small incision in the frog's abdominal wall. With an adequate recovery period, frogs can be operated upon multiple times.

Oocytes develop in a six stage process from I to VI. Stage V and VI oocytes are approximately 1-1.2 mm and possess two distinct poles—a darker-colored animal pole and a lighter, cream-colored vegetal pole. The vitelline membrane, a glycoprotein matrix which provides structural support in maintaining the oocytes' spherical shape, envelops each individual oocyte. An additional layer of follicular cells surrounds the vitelline membrane. These follicular cells are electrically coupled to one another and to the oocyte by gap junctions. As these follicular cells can interfere with recordings, they are removed prior to RNA injection and voltage or patch clamping. The collection of oocytes, follicular cells, connective tissue, and blood vessels constitutes an ovarian lobe.

3-1.1. Harvesting of Xenopus Oocytes

A single frog (Nasco, Xenopus Express, or Xenopus One) was selected for oocyte harvest through partial ovariectomy. Care was taken to select a frog whose prior surgical incisions have been allowed to heal at least 3 to 4 weeks. The frog was anesthetized in a 0.17% tricaine (3-aminobenzoic acid ethyl ester, methane sulfonate salt, Sigma) bath pH

7.4 at room temperature for approximately 10-15 minutes until it was unresponsive when held in the supine position. The frog was then placed in the supine position on a layer of Saran Wrap over a tray of ice. These measures were taken to ensure clean conditions, reduce risk of frostbite, and maintain moisture levels in the frog skin. Sterile gloves were used throughout the procedure. All surgical instruments were cleaned with 70% isopropyl alcohol prior to each use and periodically autoclaved as well. Care was taken to avoid contamination as much as possible. A small horizontal incision approximately 1 cm wide was made in the abdomen wall, lateral to the midline. Three to four ovarian lobes were gently removed from the abdominal cavity using forceps and scissors. These lobes were placed in Ca^{2+}-free ND96 solution. The wound was closed in two layers. The muscle and fascial layers were closed together with 5-0 chromic gut (Ethicon). The skin was closed with 6-0 ethilon (Ethicon). The frog was then placed into a clean individual tank for a few hours to recover from the anesthesia and surgery prior to return to the general tank.

3-1.2. Preparation, Selection, and RNA Injection of Xenopus Oocytes

The ovarian lobes were gently dissected into smaller sections using forceps. In order to separate the oocytes from the connective tissue, blood vessels, and follicular cells which together form the lobe, they were treated with collagenase A (Sigma or Boehringer Mannheim) at a concentration of 2 mg/ml of Ca^{2+}-free ND96 and gently agitated for 1 to 1.5 hours at room temperature. Any remaining follicular cells were manually removed with forceps. After incubation in collagenase, the oocytes were washed with Ca^{2+}-free ND96 to stop the digestion process and then washed with the more isotonic Ca^{2+}-plus ND96. Oocytes were stored in Ca^{2+}-plus ND96 supplemented with 450μM gentamicin (Gibco) and 2.5mM sodium pyruvate (Boehringer Mannheim) in a 18°C incubator. An alternate oocyte media based upon half-strength Leibovitz L-15 Medium (Gibco BRL) and 5mM Hepes supplemented with penicillin/streptomycin (500U/ml of media) and 50μg/ml (500μM) gentamicin was used as well.

Oocytes were selected on the basis of maturity (stage V and VI) and uniformity in the color of the animal and vegetal poles with a clear boundary between the two. Care was taken to examine the oocytes daily and to remove any cells with areas of decoloration, a speckled appearance, or outpouching of the membranes—all signs of a dying cell. The storage media was also changed every other day.

Care was taken throughout the injection protocol to minimize contamination by RNases. Gloves were worn at all times when handling the mRNA. Disposable micropipettes (Drummond Microcaps, 64mm length, 6 µl) were pulled using a pipette puller (Narishige). The end of the tip was clipped under the microscope for an opening diameter of approximately 5µm. The mRNA solution consisted of either a single type of mRNA or two types mixed together prior to injection. Concentrations of the mRNAs were 0.03 or 0.1µg/µl for ROMK1 (Kir1.1a), 0.03 or 0.1µg/µl for ROMK2 (Kir1.1b), 0.5µg/µl for CFTR, 0.5 to 0.8µg/µl for SUR1, 0.4 to 0.6µg/µl for BIR (Kir6.2), and 0.5µg/µl for A9 (a BIR/Kir6.2 mutant). For injection, the mRNA solution was transferred to a RNase-free surface. The injection pipette was attached to a hand-driven, coarse manipulator (MK1 Manipulator, Singer Instruments) which was connected to a pneumatic injector (Inject+Matic, Geneve). The droplet of mRNA solution was aspirated into the pipette as quickly as possible to avoid excessive evaporation of the microliter volumes. Pressure and time of injection were adjusted to ensure that approximately 50nl aliquots were injected into each oocyte. Exogenous mRNA was injected preferably into the vegetal pole as the voltage clamp microelectrodes were inserted into the animal pole during experiments.

3-2. Molecular Biology

3-2.1. Bacterial Cell Transformation and Plasmid Purification

Subcloning efficiency DH-5α or DM1 competent Escherichia coli cells were used for bacterial cell transformation. Approximately 500ng of plasmid DNA was added to

100µl of competent cells in a chilled microcentrifuge tube and incubated on ice for 30 minutes. The mixture was heat shocked for 45 seconds at 37°C before being placed back on ice for 2 minutes. 0.9ml of Luria-Bertani (LB) media without antibiotics (ampicillin) was added to the microcentrifuge tube and then the E. coli/plasmid DNA mixture was grown on a shaker for 1 hour to overnight at 37°C. A series of dilutions of the transformation growup was plated on LB-ampicillin (100 µg/ml) agar plates and then incubated at 37°C overnight. Ampicillin was used to apply selection pressure ensuring that only daughter cells containing the plasmid of interest remain. A single bacterial colony from the LB-ampicillin plates was used for growup. Depending on the plasmid purification kit being used (Qiagen Midi-prep, 5 Prime→3 Prime BIGprep, or 5 Prime→3 Prime PERFECTprep), the single colony was inoculated in 5-60 ml of LB buffer plus ampicillin (100 µg/ml) and shaken overnight at 37°C. Alternate "richer" growth media such as Terrific Broth (TB) can be used as well. Plasmid purification was performed per the protocols provided in the various kits.

The basic principle of plasmid purification is lysis of bacterial cells followed by purification and isolation of supercoiled plasmid DNA. The amount of culture volume processed in each sample is dependent upon the cell density. If the volume is too large there may be inefficient cell lysis, overload of the binding column, and a high lysate viscosity. Care must be taken to mix the lysate thoroughly but gently to avoid shearing of the bacterial genomic DNA whose fragments may contaminate the plasmid DNA. The lysate is applied to a resin column where the plasmid DNA is selectively bound. The column is washed with buffers to remove RNA and protein contaminants. The plasmid DNA is then eluted from the column, desalted, concentrated, washed, and finally resuspended in RNase-free H_2O. The plasmid DNA is stored at -20°C.

3-2.2. Linearization of cDNA

Basic principles of setting up a restriction reaction include: 1) choosing an appropriate restriction enzyme, 2) determining the concentration of the enzyme, 3) determining the minimum amount of enzyme required for effective linearization, 4) determining the concentration of the plasmid DNA, 5) deciding upon the amount of DNA to be linearized, 6) deciding upon the desired excess of enzyme, and 7) calculating the amount of buffer and other solutions needed for optimal action of the specific restriction enzyme. The restriction reaction is incubated for 1.5 to 2 hours or overnight at 37°C. After an appropriate period of incubation, proteinase K and sodium dodecyl sulfate (SDS) are added to halt the restriction reaction, digest the remaining restriction enzyme, and eliminate RNase contamination. Care is taken from this point to remain RNase-free. The plasmid DNA is extracted with a 25:24:1 (vol.) phenol:chloroform:isoamyl alcohol mixture. The phenol serves to denature proteins in the nucleic acid solution. Subsequent chloroform extractions serve to remove residual phenol from the aqueous phase containing the linearized DNA. Together, these extractions also remove residual SDS which may interfere with in vitro transcription reactions. Ammonium acetate and ethanol are added to precipitate the nucleic acid and to reduce coprecipitation of unincorporated nucleotides. The precipitate is washed in ethanol to remove residual salts and ethylenediamine-tetraacetic acid (EDTA) which may later interfere with bacteriophage RNA polymerases. Finally, it is microcentrifuged to pellet the DNA and allowed to dry prior to resuspension of the linearized DNA in RNase-free dH_2O and stored at -20°C.

Human SUR1 subcloned into PCR3.1 or Bluescript M13 was provided by Dr. Pamela Thomas (University of Michigan, Ann Arbor). SUR1 was linearized using Xba I restriction enzyme (New England BioLabs) for cleavage at the carboxy-terminal (3') end.

Human BIR (Kir6.2) subcloned into PCR2.1 was provided by Dr. Pamela Thomas (University of Michigan, Ann Arbor). BIR (Kir6.2) was linearized using Spe I restriction enzyme (New England BioLabs).

Human CFTR subcloned into pBQ4.7 was provided by Drs. William B. Guggino and Erik M. Schwiebert (Johns Hopkins University, Baltimore and University of Alabama Birmingham, Birmingham). CFTR was linearized using EcoR V restriction enzyme (New England BioLabs).

Human ROMK2 (Kir1.1b) subcloned into pSport1 was provided by Drs. Gerhard Giebisch (Yale University, New Haven) and Steven C. Hebert (Vanderbilt University, Nashville). ROMK2 (Kir1.1b) was linearized using Not I restriction enzyme (New England BioLabs).

3-2.3. *In Vitro* Transcription of Capped mRNA

In vitro transcription was performed using the Ambion MEGAscript or mMessage mMachine kits to produce capped mRNA for Xenopus oocyte injection purposes. Capping of mRNA allows for more efficient translation. Each kit includes the appropriate bacteriophage RNA polymerase (SP6, T7, or T3) as well as transcription buffer, enzyme mix, ribonucleotide solutions, DNase, transcription stop mix, and lithium chloride precipitation solution.

The general principles of *in vitro* transcription involve the assembly of a transcription reaction which consists of linearized template DNA, ribonucleotides, cap analog, RNA polymerase, and transcription buffer. This transcription reaction is incubated at 37°C for 1 to 6 hours depending on the length of the transcript and the transcription kit being used. DNase 1 is added to stop transcription and degrade the template DNA. The mRNA is then precipitated by lithium chloride and washed by ethanol to remove unincorporated nucleotides. An alternative method is phenol/chloroform extraction followed by precipitation with ammonium acetate and isopropyl alcohol, a process which may be more effective in removal of proteins. Both methods end with centrifugation to pellet the mRNA, careful removal of the supernatant, and resuspension of the mRNA in RNase-free dH_2O. The mRNA was stored at -80°C.

3-2.4. Verification of cDNA and mRNA by Gel Electrophoresis

Care was taken to verify that the appropriate plasmid DNA had been produced during the bacterial growup and subsequent plasmid purification process by 1% agarose gel electrophoresis. Prior to extraction and purification of the linearized cDNA, gel electrophoresis was repeated to confirm successful linearization of the cDNA in the restriction reaction. As the mRNA produced by *in vitro* transcription is sensitive to RNases present in the environment, the quality of the mRNA is confirmed by gel electrophoresis prior to injection into oocytes.

3-3. Two-Electrode Voltage Clamp Technique

3-3.1. Theory

Voltage clamp studies are based upon the principle of setting the membrane voltage and measuring the transmembrane current require to maintain that voltage (Sherman-Gold, 1993). By clamping the voltage, the capacitive spike/transient is effectively limited to the brief time required to charge the membrane prior to changing the membrane to the set voltage. In doing so, the current effectively becomes proportional to the membrane conductance which is determined by the number of open channels.

The intracellular microelectrodes used in two-electrode voltage clamping consist of a AgCl-coated Ag wire placed within a solution-filled glass micropipette. To reduce electrode resistance concentrated KCl solution can be used. While such a solution also allows more accurate measurement of resting potential, it has the disadvantage of potentially altering the normal ion concentrations within the cell as a result of the fluid connection which exists between the electrode and the cell interior. Smaller pipette tip diameters would minimize such mixing; however, the concomitant increase in the resistance of the pipette becomes the limiting factor as it impacts on current passing ability and noise.

Selection of the glass used for the intracellular microelectrodes is dependent upon the magnitude of currents to be measured, the degree of "acceptable" noise, and the need to generate a tight seal. Thickness of the walls determines the degree of electrical noise generated and the bluntness at the tip. Other properties of the glass to consider are the temperature required to soften the glass and the noise that it will generate. For the purposes of two-electrode voltage clamping, sharper tips on the standard intracellular microelectrodes are required in order to penetrate the cell membrane. For patch clamp experiments, glass selection is more critical in order to achieve gigaohm seals. Our micropipettes were pulled in a vertical multiple stage Narishige pipette puller.

Voltage clamping oocytes poses two unique challenges due to the size of the cell and its relative efficiency in expressing injected mRNA. In stage V and VI oocytes, the surface area, assuming an ideal spherical shape and not taking into account invagination of the membrane, is on the order of 10^6 μm^2. As capacitance is proportional to area, the current necessary to charge up the membrane prior to changing the membrane potential is significant and consequently the time to charge the membrane is longer. In addition, the large currents which may result from efficient channel protein expression and insertion into the cell membrane may pose problems in controlling the voltage. To address the problem of such a large membrane capacitance, lower resistance electrodes can be utilized. Appropriate shielding of the electrodes to reduce capacitive coupling also improves the speed at which voltage changes can be achieved.

3-3.2. Channel Recording and Analysis

Data was acquired through the GeneClamp 500 (Axon Instruments) with conversion of the continuous analog data into digital format through the Clampex program within the pCLAMP6 suite (Axon Instruments). The Clampex program set the membrane at various holding potentials while simultaneously recording the transmembrane current. At specified time points—one minute intervals for our experiments—a trial was completed

and the data acquired was saved to a data file. Data was viewed on a monitor (Gateway 2000 Vivitron 1776). Recordings were exported to computer (Gateway 2000 P5-166) through the Clampfit program and then converted to spreadsheet format in Microsoft Excel for analysis.

3-4. Protocol

3-4.1. Rationale for Use of Potassium Channel Blockers and Openers

A number of pharmacologic agents were used in the experiments including sulfonylureas, barium, diazoxide, and sodium azide. The sulfonylureas, oral hypoglycemics used in the treatment of NIDDM, are of interest in our studies because of the high sensitivity of SUR1 to these compounds as well as their interactions with CFTR, another member of the ABC transporter family. A variety of different sulfonylureas (**glibenclamide**, tolbutamide, etc.) are currently available. They are lipophilic molecules and appear to be effective in blocking K_{ATP} current from either side of the membrane, perhaps as a result of diffusion across the membrane. The other potassium channel blocker used in our experiments is barium. **Barium** has been demonstrated to block a variety of potassium channels (voltage-gated, inwardly rectifying, ATP-sensitive). This suggests that there may be similarity between the pore regions of these potassium channels.

Diazoxide, a benzothiadiazine structurally similar to the sulfonylureas, is an effective opener of the potassium channel in pancreatic beta cells. It produces membrane repolarization and consequently inhibition of insulin secretion. It also appears to be effective from either side of the membrane and dependent upon both ATP and Mg^{2+} for its action. The exact mechanism by which diazoxide activates K_{ATP} channels is unclear. **Sodium azide** is a metabolic inhibitor of mitochondrial electron transport protein cytochrome a_3 and the F_1/F_0 ATPase. Sodium azide brings about a decrease in intracellular ATP levels which presumably allows for an activation of the BIR (Kir6.2)-SUR1 channel complex and subsequent potassium currents.

3-4.2. Protocol for ROMK-Coinjected Oocytes

Two-electrode voltage clamp experiments measuring whole-cell currents and response to sulfonylureas were performed on Xenopus oocytes injected with ROMK1 (Kir1.1a) alone, ROMK2 (Kir1.1b) alone, ROMK1 (Kir1.1a) plus CFTR or SUR1, and ROMK2 (Kir1.1b) plus CFTR or SUR1. Experiments were performed on days 2-6 post-injection to ensure adequate time for channel protein expression. Experiments were conducted at room temperature, 20-22°C. All oocytes were maintained in 0.01 K^+ or 0.1 K^+ bath solution prior to the start of each experiment. For each experiment, micropipettes (Kimax-51, Kimble Products) with typical resistances of 0.5-1.5 MΩ were used. Resistances were checked prior to each experiment and no set of micropipettes was used for more than three consecutive experiments.

For experiments where ROMK1 (Kir1.1a) or ROMK2 (Kir1.1b) where expressed alone, oocytes were preincubated in 0.01 K^+ bath solution for 10 minutes. Initial baseline current readings were obtained in this bath solution. Following impalement of the oocyte with both microelectrodes, the resting membrane potential was measured prior to clamping the membrane at a specified holding potential (typically -65mV). The experimental protocol consisted of current-voltage measurements (a trial) recorded at 60 second intervals in various bath solutions. Each trial consisted of as series of 20ms test pulses starting at -100mV increasing in 20mV increments up to a final voltage of +40mV or +100mV. The protocol began with a 5 minute equilibration period in 0.01 K^+ bath solution followed by a 14 minute test of sulfonylurea (glibenclamide) sensitivity. After assessing the response to glibenclamide, barium chloride (2mM) was then added to the bath solution for 2 minutes to determine the extent to which the current was dependent upon potassium movement. A final 5 minute recovery period in 0.01 K^+ bath solution concluded the experiment.

For experiments in ROMK1 (Kir1.1a)-CFTR and ROMK2 (Kir1.1b)-CFTR coexpressed oocytes, the above protocol was also used.

For experiments in ROMK1-SUR and ROMK2 (Kir1.1b)-SUR coexpressed oocytes, the protocol was modified as follows. During set-up of the voltage clamp, the oocytes were transferred out of the Ca^{2+}-plus ND96 storage media into 0.1 K^+ or 0.5 K^+ bath solution for approximately 3 to 5 minutes to allow the oocyte to re-equilibrate in the lower potassium solution. The protocol consisted of 5 minutes in 0.1 K^+ or 0.5 K^+ bath solution followed by 5 to 7 minutes in 100μM or 250μM glibenclamide, 5 minutes recovery period in 0.1 K^+ or 0.5 K^+ bath solution, 2 to 3 minutes in 2mM barium chloride, and a final wash in 0.1 K^+ or 0.5 K^+ bath solution.

Control experiments in uninjected oocytes were subject to the same protocols as described above.

3-4.3. Protocol for BIR-Coinjected Oocytes

Two-electrode voltage clamp experiments measuring whole-cell currents and response to sulfonylureas were performed on Xenopus oocytes injected with BIR (Kir6.2) alone, SUR1 alone, BIR (Kir6.2) plus SUR1, and A9 (BIR/Kir6.2 mutant) plus SUR1. Conditions for experiments were as above (Section 3-4.2).

Experiments were initially performed on oocytes injected with BIR (Kir6.2) alone. BIR (Kir6.2) is postulated to be the pore subunit of the pancreatic beta cell K_{ATP} channel. To assess the level of channel activity present when expressed alone experiments were done exposing the oocyte first to standard ND96 (2mM KCl) bath solution then to High K^+ (90mM KCl) bath solution. As we did not expect variations in the intracellular ATP concentration to result in BIR (Kir6.2) activation, no preliminary experiments with sodium azide were done.

Next, to confirm that SUR1 expressed alone would not result in potassium current and to assess if SUR1 could couple with any endogenous oocyte channels, experiments were done exposing the oocyte first to standard ND96 (2mM KCl) bath solution then to High K^+ (90mM KCl) bath solution. As a member of the ABC transporter superfamily,

SUR1 is responsive to changes in intracellular ATP concentrations. To assess if sodium azide metabolic inhibition of the oocyte would cause channel activation, experiments were done exposing the oocyte for 5 minutes to High K^+ bath solution then 15 minutes to 3mM sodium azide.

Lastly, we examined oocytes coexpressing BIR (Kir6.2)-SUR1. The protocol for these coinjected oocytes was 7 minutes in High K^+ bath solution followed by 16 to 17 minutes in High K^+ bath solution plus 340μM diazoxide and 5mM sodium azide.

For oocytes coinjected with SUR1 and the BIR (Kir6.2) mutant A9, the protocol used was 3 minutes in High K^+ bath solution followed by 15 minutes in High K^+ bath solution plus 340μM diazoxide and 5mM sodium azide. To assess if duration of exposure to diazoxide and sodium azide was adequate preincubation experiments were done as well. The protocol for these experiments consisted of 5 to 10 minutes of preincubation in High K^+ bath solution plus 340μM diazoxide and 5mM sodium azide followed by 10 minutes of current-voltage measurements recorded at 60 second intervals in the same bath solution. As for the experiments described above, each trial consisted of 20ms test pulses starting at -100mV increasing in 20mV increments up to +40mV or +100mV.

Solutions and Chemicals

Ca^{2+}-plus ND96 Solution: 96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes

Ca^{2+}-free ND96 Solution: 82.5mM NaCl, 2mM KCl, 1.8mM $MgCl_2$, 5mM Hepes

Standard (ND96) Bath Solution: 96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes

High K^+ Bath Solution: 90mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 5mM Hepes

0 K^+ Bath Solution: 105mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes

0.01 K^+ Bath Solution: Prepared by addition of 0.33μl of 3M KCl per 100ml of 0 K^+ Bath Solution

0.1 K^+ Bath Solution: 105mM NaCl, 0.1mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes

0.5 K^+ Bath Solution: 105mM NaCl, 0.5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes

Pipette Solution: 3M KCl

LB plates were made from 10g Bacto-Tryptone, 5g yeast extract, 5g NaCl, 2ml of 2N NaOH, 15g Bacto-Agar, and nano-H_20 to 1000 ml. This mixture was autoclaved and cooled to approximately 40°C prior to the addition of ampicillin (100µg/ml) and then poured into plates. LB buffer was made from 10g Bacto-Tryptone, 5g yeast extract, 5g NaCl, 1ml of 1M $MgSO_4$, 10 ml of 1M Tris, and nano-H_20 to 1000 ml. This mixture was autoclaved prior to storage at 4°C. Ampicillin stock (100mg/ml) was made in sterile RNase-free dH_2O and stored at -20°C until needed for LB-ampicillin plates or for bacterial growups.

Glibenclamide (sulfonylurea) was dissolved in a 2:1 (vol.) ethanol:dimethyl sulfoxide (DMSO) mixture to make a 0.001M or 0.1M stock solution. The stock solution was stored at 4°C. A 100nM, 100µM, or 0.5mM working solution was made as needed for experiments; any remaining working solution was discarded after the second day of experiments. Glibenclamide was obtained from Sigma.

Barium chloride was dissolved in 0 K^+ bath solution to make a 0.1M stock solution . The stock solution was stored at 4°C. A 2mM or 5mM working solution was made as needed for experiments; any remaining working solution was discarded after two weeks of experiments. Barium chloride was obtained from Sigma.

Diazoxide was dissolved in standard bath solution to make a 68mM stock solution . The stock solution was stored at 4°C. A 340µM working solution (Gribble et al., 1997) was made as needed for experiments; any remaining working solution was discarded at the end of each day. Diazoxide was obtained from Sigma.

Sodium azide was dissolved in standard bath solution to make a 3M stock solution . The stock solution was stored at 4°C. A 3mM or 5mM working solution was made as needed for experiments; any remaining working solution was discarded at the end of each day. Sodium azide was obtained from Sigma.

The pH of each solution was adjusted to 7.4 with the exception of the diazoxide and sodium azide solution which was adjusted to a range of 7.4 to 7.6 as the diazoxide is insoluble in water but soluble in alkaline solutions.

3-5. Criteria for Inclusion of ROMK Injected Oocytes in Data Analysis Set

Oocytes were evaluated for level of channel expression. If initial baseline currents were less than 500nA, the experiment was excluded for poor expression. An exception was made for oocytes injected with 0.03μg/μl of mRNA; current readings of less than 500nA were accepted because of the low mRNA concentration. Only oocytes which maintained a stable baseline during the first 5 to 7 minutes in control bath solution were included in our study. A stable baseline was defined as a variation in current measurements of less than 10 percent for the entire 5 to 7 minute period. If the oocyte met the above criteria, an average baseline current was calculated over a 2 minute interval. To assess KATP sensitivity, an average current was also calculated over a 2 minute interval for an end-glibenclamide current reading. The percent inhibition by glibenclamide was calculated from the change in current ($I=I_{baseline}-I_{end\text{-}glibencamide}$) over total current. Total potassium current was calculated from glibenclamide-sensitive and barium-sensitive currents.

3-6. Statistical Analysis

To determine the statistical significance of observed differences in the glibenclamide sensitivity of the ROMK channels when expressed alone or in the presence of an ABC transporter, we employed both the student t-test and the Kruskal-Wallis one way analysis of variance on ranks (ANOVA). The student t-test was employed when comparing two experimental groups. The ANOVA was employed when comparing among greater than two experimental groups.

4. Results

4-1. ROMK Injected Oocytes

4-1.1. ROMK2-Coinjected Oocytes

We postulate that direct interactions between K_{ATP} channels and ABC transporters are the mechanism by which ABC transporters serve as regulators of channel activity. Therefore, changes in the structure of either the channel protein or the ABC transporter would be expected to impact upon their ability to couple and upon channel activity. Previously we have identified that an intact first nucleotide-binding fold (NBF) is necessary for the interaction between CFTR and ROMK2 (Kir1.1b). If, in fact, it is true that these protein-protein interactions are dependent upon a domain common to all ABC transporters, such as the NBF, then wild-type SUR1 with intact NBFs should be able to couple with ROMK2 (Kir1.1b) and confer sulfonylurea sensitivity. To examine this hypothesis, we conducted two-electrode voltage clamp experiments on Xenopus oocytes injected with ROMK2 (Kir1.1b) alone, ROMK2 (Kir1.1b)-CFTR, and ROMK2 (Kir1.1b)-SUR1 to assess sulfonylurea sensitivity of the K_{ATP} channel alone and in the presence of an ABC transporter. Resting baseline currents were measured during the 5 to 7 minute equilibration period in control $0.01K^+$ bath solution. The final experimental protocol consisted of this equilibration period followed by a 14 to 15 minute exposure to 500μM glibenclamide, a 2 to 3 minute exposure to 2mM barium chloride to assess total potassium current, and a final 5 minute recovery period in control bath solution. For some of the ROMK2 (Kir1.1b)-SUR1 injected oocytes (n=3), an alternate protocol was used initially. In this protocol, oocytes had a 5 minute equilibration period in a control $0.1K^+$ bath, a 7 minute exposure to 100μM glibenclamide, a 5 minute wash in control bath, a 2 minute exposure to 2mM barium chloride, and finally a 5 minute recovery in control bath. These initial experiments demonstrated that the effects of glibenclamide inhibition continued into the wash period. As a result the protocol was modified; the glibenclamide concentration was increased, the

time of exposure to glibenclamide was increased, and the post-glibenclamide wash period was eliminated.

Table 3 and Figure 7 summarize the data from oocytes injected with ROMK2 (Kir1.1b) alone, ROMK2 (Kir1.1b)-CFTR and ROMK2 (Kir1.1b)-SUR1. The potassium currents observed in oocytes injected with ROMK2 (Kir1.1b) alone (n=11) decreased by 23.1±6.1% inhibition after exposure to glibenclamide. In contrast, the similar currents in oocytes expressing ROMK2 (Kir1.1b) with either ABC transporter showed significantly increased glibenclamide sensitivity (p=0.01). Coexpression of ROMK2 (Kir1.1b)-CFTR (n=11) and ROMK2 (Kir1.1b)-SUR1 (n=13) resulted in 54.8±10.8% and 56.5±6.7% decrease in potassium current in the presence of glibenclamide inhibition, respectively. This data supports the hypothesis that both of these ABC transporters with intact NBFs were able to interact with ROMK2 (Kir1.1b) resulting in an enhanced sensitivity to glibenclamide. In addition, the difference in glibenclamide inhibition between ROMK2 (Kir1.1b)-CFTR and ROMK2 (Kir1.1b)-SUR1 was not found to be statistically significant (p=0.89).

Figure 8 depicts a representative time-course for an experiment in an oocyte expressing ROMK2 (Kir1.1b)-SUR1 as well as individual current-voltage traces at time points throughout the experiment.

4-1.2. ROMK1-Coinjected Oocytes

Having determined that the NBF1 of ABC transporters is involved in protein-protein interactions with ROMK2 (Kir1.1b), we directed our attention to the "associated" K_{ATP} channel itself. To examine if there are domains within the K_{ATP} channel itself important for coupling to occur, we studied N-terminus modifications in ROMK. Two-electrode voltage clamp experiments were conducted on Xenopus oocytes injected with ROMK1 (Kir1.1a) alone, ROMK1 (Kir1.1a)-CFTR, and ROMK1 (Kir1.1a)-SUR1 to determine if there was any change in sulfonylurea sensitivity when the K_{ATP} channel was

expressed alone versus in the presence of an ABC transporter. The protocol for these oocytes was as described above. Again, the alternate protocol was used initially for a few of the ROMK1-SUR1 coinjected oocytes (n=2).

Table 3 and Figure 9 summarize the data from oocytes injected with ROMK1 (Kir1.1a) alone, ROMK1 (Kir1.1a)-CFTR and ROMK1 (Kir1.1a)-SUR1. Oocytes injected with ROMK1 (Kir1.1a) alone (n=8) demonstrated mild to moderate inhibition (28±2.8%) of potassium currents after exposure to glibenclamide. Oocytes injected with ROMK1 (Kir1.1a)-SUR1 (n=6) demonstrated a similar degree of glibenclamide inhibition (24.6±6.3%, p=0.6) suggesting that coexpression of these two subunits did not result in an interaction capable of altering the sulfonylurea sensitivity. These findings can be contrasted to those from oocytes coexpressing ROMK2 (Kir1.1b)-SUR1 where there is an enhanced sulfonylurea sensitivity. Interestingly, in oocytes injected with ROMK1 (Kir1.1a)-CFTR (n=6) the currents were significantly less sensitive to glibenclamide, with only of 6±0.02% (p=0.009) inhibition of the total potassium current when compared to either ROMK1 (Kir1.1a) alone or ROMK1 (Kir1.1a)-SUR1 potassium currents.

4-2. BIR Injected Oocytes

To examine the normal coupling interactions of SUR1 and BIR (Kir6.2) and the effects of mutations in the M2 region of the channel pore upon this interaction, experiments were performed on oocytes expressing BIR (Kir6.2)-SUR1, A9 (a BIR/Kir6.2 mutant)-SUR1, as well as SUR1 and BIR (Kir6.2) alone.

4-2.1. Expression of BIR and SUR1 Alone

BIR (Kir6.2) is postulated to be the pore subunit of the pancreatic beta cell K_{ATP} channel. Two-electrode voltage clamp experiments were initially performed on Xenopus oocytes injected with BIR (Kir6.2) alone (n=3/5) to determine if this subunit resulted in channel activity. These oocytes were placed in bath solutions with varying potassium

concentrations and channel activity was measured. Current recordings were made in standard ND96 (2mM KCl) bath solution then High K^+ (90mM KCl) bath solution. When placed in the High K^+ bath, oocytes demonstrated a slight increase in potassium current as would be expected from the greater electrochemical potassium gradient. However, this change in potassium current was similar to those measured in uninjected oocytes (n=8) or H_2O-injected oocytes (n=5). These results suggested that the BIR (Kir6.2) subunit expressed alone did not result in a functional potassium channel.

Next we turned our attention to SUR1 which is postulated to be the regulatory subunit and ATP sensor of the pancreatic beta cell K_{ATP} channel. We repeated the above protocol in oocytes expressing SUR1 alone (n=5) to confirm that SUR1 was not a potassium channel itself and to determine if SUR1 would couple with an endogenous K_{ir} channel. Potassium currents were found to be similar to those measured in uninjected oocytes in these experiments as well. As a member of the ABC transporter superfamily, SUR1 possesses two NBFs allowing it to be responsive to changes in intracellular ATP concentrations. To assess if sodium azide metabolic inhibition of the oocyte would cause channel activation, experiments were done exposing the SUR1-injected oocytes (n=3) to High K^+ bath solution for 5 minutes then to 3mM sodium azide for 15 minutes. Under these experimental conditions, oocytes injected with SUR1 alone did not demonstrate an appreciable increase in potassium currents.

The result of our experiments in oocytes expressing BIR (Kir6.2) or SUR1 alone confirmed those reported by Gribble et al. demonstrating that coexpression of both subunits was necessary to reconstitute K_{ATP} currents.

4-2.2. Coexpression of BIR and SUR1

To assess the normal coupling of both K_{ATP} channel subunits, we examined oocytes coexpressing BIR (Kir6.2)-SUR1. In these experiments we exposed the coinjected oocytes to a High K^+ solution followed by a High K^+ solution plus 340μM diazoxide and

5mM sodium azide. Treatment with diazoxide and sodium azide resulted in potassium currents significantly greater than measured in High K^+ solution alone. We were able to demonstrate this in 2 oocytes confirming the results reported by Gribble et al. However, we experienced some difficulty in repeating this result consistently. Whether this reflects problems with the Xenopus oocyte as a reliable expression system or is an issue with the quality of the mRNA has not been determined. In the oocytes that did express, however, the average currents measured at -100mV were approximately -8 to -40μA which are similar to those previously reported (Gribble et al., 1997).

4-2.3. Coexpression of a BIR mutant and SUR1

Having confirmed that the Xenopus oocyte expression system was adequate for studying interactions between BIR (Kir6.2) and SUR1 we examined oocytes coinjected with a BIR/Kir6.2 mutant (A9) and SUR1. Our goal was to determine if potassium currents could be stimulated in these oocytes. Oocytes were equilibrated for 3 minutes in High K^+ bath solution then exposed to High K^+ bath solution plus 340μM diazoxide and 5mM sodium azide for 15 minutes. No change in potassium current was observed (n=2). To assess if duration of exposure to diazoxide and sodium azide was adequate, preincubation experiments were done as well. The protocol for these experiments consisted of 5 to 10 minutes of preincubation in High K^+ bath solution plus 340μM diazoxide and 5mM sodium azide followed by 10 minutes of current-voltage measurements recorded at 60 second intervals in the same bath solution. Again, no change in potassium current was observed (n=5). These preliminary findings appear to suggest that coexpression of the BIR (Kir6.2) mutant A9 with SUR1 does not result in a functional K_{ATP} channel and that altering the M2 region of the channel pore leads to a dysfunctional channel.

5. Discussion

5-1. Effects of Mutations in CFTR upon Interactions with ROMK2

A recent study by McNicholas et al. suggested that mutations in specific domains of CFTR—the first nucleotide-binding fold (NBF1)—interfere with CFTR's ability to interact with the ROMK2 (Kir1.1b) channel (McNicholas et al., 1997). In that study glibenclamide was shown to inhibit 56% of potassium current in oocytes expressing both ROMK2 (Kir1.1b) and CFTR. In contrast, there was only 11% inhibition of potassium currents in oocytes expressing ROMK2 (Kir1.1b) alone. Furthermore, when ROMK2 (Kir1.1b) was expressed with CFTR constructs containing mutations in the first nucleotide-binding fold (NBF1), there was no enhancement of glibenclamide sensitivity suggesting that an intact NBF1 is necessary for this interaction. Supporting this hypothesis, oocytes injected with ROMK2 (Kir1.1b) and a truncated CFTR construct containing an intact NBF1 still exhibited enhanced (46%) glibenclamide sensitivity. In contrast, when ROMK2 (Kir1.1b) was coexpressed with a truncated CFTR construct lacking NBF1 only 12% inhibition remained. Given that NBFs are motifs common to ABC transporters these data suggest that certain family members may be able to substitutes for other members.

5-2. Ability of SUR1 to Substitute for CFTR in Interactions with ROMK2

Pursuing this theory further, we wondered if SUR1 could substitute in the ROMK2 (Kir1.1b)-CFTR interaction. Studying SUR1's ability to substitute for CFTR in its interactions with the ROMK family of channel proteins offered the opportunity to examine similarities and differences in the domain organization of these two ABC transporters (Figure 10). In our studies, oocytes coexpressing either ROMK2 (Kir1.1b)-CFTR or ROMK2 (Kir1.1b)-SUR1 demonstrated enhanced sensitivity to sulfonylureas when compare to ROMK2 (Kir1.1b) alone. In addition, when compared between themselves, the degree to which these two ABC transporters were able to enhance glibenclamide

sensitivity was not statistically significant. These data not only demonstrate that it may be possible for one family member to substitute for another and provide similar regulatory function but also suggest that similarities exist in how CFTR and SUR1 interact with ROMK2 (Kir1.1b). Furthermore, it implicates the NBF as an essential component for this interaction. As the NBF is the region of the protein that binds ATP, one can speculate that this interaction may be altered by energy dependent processes such as phosphorylation-dephosphorylation. Additional studies focusing upon these questions will need to be done.

5-3. Effects of Alterations in ROMK upon Interactions with CFTR

Clearly changes in the ABC transporter impact upon its ability to interact with heterologous channel proteins. If, as we postulated, the relationship between ABC transporters and channels are dynamic involving direct protein-protein interactions, we would expect that changes in the channel itself would also effect these interactions. Specifically, we postulated that changes in the cytoplasmic N-terminus of ROMK would alter its interaction with CFTR. Our choice to study the N-terminus was based upon studies by Tinker et al. and Tucker et al. Tinker et al. suggested that assembly of a functional multimeric K_{ir} channel is dependent upon compatibility of the proximal C-terminus and the M2 region (Tinker et al., 1997). Tucker et al. demonstrated that BIR (Kir6.2) mutants with C-terminus deletions expressed channel activity when injected alone into Xenopus oocytes while those with N-terminus deletions did not express channel activity, suggesting that the C-terminus may inhibit channel activity by blocking the pore (Tucker et al., 1997). When the C-terminus truncated forms of BIR (Kir6.2) were coexpressed with SUR1, Tucker et al. found enhancement of ATP sensitivity as well as restoration of the effects of diazoxide, tolbutamide, and Mg-ADP, suggesting that SUR1 coupling is not affected by the C-terminus deletions. In addition, studies (Tinker et al., 1997) have shown the N-terminus and M1 region to be important in the assembly of functional multimeric voltage-gated potassium channels, suggesting that the N-terminus is

capable of playing a role in protein-protein interactions. Our studies, as described earlier, had confirmed that a ROMK2 (Kir1.1b)-CFTR interaction exists. To examine if alterations in the N-terminus of ROMK2 (Kir1.1b) would impact upon CFTR's ability to increase inhibition of ROMK2 (Kir1.1b) by glibenclamide, we studied another isoform of the ROMK channel, ROMK1 (Kir1.1a) whose N-terminus had an additional 19 amino acids (Figure 11). Both isoforms, ROMK1 (Kir1.1a) and ROMK2 (Kir1.1b), express functional K_{ir} channels when expressed alone. We found that oocytes expressing ROMK1 (Kir1.1a)-CFTR did not exhibit the enhanced glibenclamide sensitivity we had seen in oocytes expressing ROMK2 (Kir1.1b)-CFTR. In fact, there was a statistically significant decrease in glibenclamide inhibition of the potassium current as compared to oocytes with ROMK1 (Kir1.1a) alone, suggesting that there was an antagonistic effect on glibenclamide sensitivity. There are a number of possible explanations for the decrease in sensitivity. One possible explanation is that there was no coupling between CFTR and ROMK1 (Kir1.1a) and that the decreased effect of glibenclamide, although statistically significant, is not physiologically relevant. Another explanation may be that some coupling of the two subunits occurs which interferes with sulfonylurea inhibition of channel activity. Further studies will need to be done to address this issue. However, these data suggest that alterations in the N-terminus of the K_{ir} channel subunit also effect the ABC transporter-channel protein interaction.

5-4. Effects of Alterations in ROMK upon Interactions with SUR1

To assess if the ROMK1 (Kir1.1a)-CFTR findings describe a real interaction with inhibitory effects upon glibenclamide sensitivity or reflect a lack of coupling between the two subunits, we examined another ABC transporter, SUR1. In oocytes coexpressing ROMK1 (Kir1.1a)-SUR1, there was neither increase nor decrease of glibenclamide inhibition compared to ROMK1 (Kir1.1a) alone suggesting that no interaction occurred. The simplest explanation of this finding is that the results observed with ROMK1

(Kir1.1a)-CFTR represent a non-interactive state as well and that statistical significance was a function of the statistical analysis.

If one examines ROMK1 (Kir1.1a) versus ROMK2 (Kir1.1b), the 19 amino acid difference is in an region that has been shown to have a number of PKC sites. Thus, the alteration in the interaction may be dependent on a number of factors: changes in PKC activity, specifically the phosphorylation or dephosphorylation states of the channel, and/or conformational changes. Further studies will need to be done.

5-5. Effect of a Mutation in BIR upon Interactions with SUR1

Previous studies have shown that mutations within the SUR1 subunit have resulted in cases of PHHI, presumably through decreased K_{ATP} channel activity and subsequent oversecretion of insulin (Thomas et al., 1995; Aguilar-Bryan and Bryan, 1996; Kane et al., 1996; Thomas et al., 1996; Dunne et al., 1997). Specifically, mutations in NBF2 and NBF1 have been implicated. As SUR1 with its NBFs is believed to be responsible for sensing ATP levels within the pancreatic beta cell, mutations affecting the interaction between the NBF and ATP could result in the clinical features of PHHI. BIR (Kir6.2) is believed to be the predominant pore-forming subunit of the pancreatic beta cell K_{ATP} channel. However, BIR (Kir6.2) expressed alone within the Xenopus oocyte expression system does not produce K_{ATP} channel currents and appears to be inactive. A recent study by Thomas et al. reported a mutation in BIR (Kir6.2) which is predicted to disrupt the second transmembrane domain of the K_{IR} channel. This mutation was found in a child affected with PHHI, with clinical features that did not appear to differ from cases where the mutation was found to be present in SUR1. In our study we examined yet another BIR (Kir6.2) mutant, A9, which is postulated to produce a mutation of the M2 region of BIR (Kir6.2).

In our study we were able to replicate in the Xenopus oocyte expression system some of the results reported in 1997 by Gribble et al. Expression of either BIR (Kir6.2) or

SUR1 alone did not reconstitute K_{ATP} currents. In two oocytes coexpressing BIR (Kir6.2) and SUR1 we were able to demonstrate reconstitution of this current when stimulated with diazoxide and sodium azide. When oocytes coexpressing the BIR (Kir6.2) mutant A9 and SUR1 were measured, current readings were similar to those of uninjected or H_2O-injected oocytes. This suggested that the interaction between the BIR (Kir6.2) and SUR1 subunits was somehow disrupted as a result of the mutation in A9.

As the interaction between SUR1 and BIR (Kir6.2) is still unclear there are a number of mechanisms by which this disruption could occur. Our hypothesis was that direct interactions are responsible for SUR1's regulation of BIR (Kir6.2) and that mutations affecting the ability of the two subunits to interact would result in K_{ATP} dysfunction and the presentation of PHHI. It is also possible that the A9 mutation alters the processing of the K_{ir} subunit making it less available to interact with SUR1. Since the stoichiometry of the relationship between SUR1 and BIR (Kir6.2) in order for reconstitution of a function K_{ATP} channel also has not been elucidated this is an area that needs further investigation.

Alternatively, a recent study by Shyng et al. examined the effect of mutations of pore-lining residues upon stability of the open channel and suggested that structural changes within the BIR (Kir6.2) subunit may impact upon ATP-sensitivity of the channel (Shyng et al., 1997). Another mechanism by which mutations in BIR (Kir6.2) may result in an inactive K_{ATP} channel is through alterations in either the conformation or structure of the pore-lining residues.

5-6. Clinical Implications

As defects in ion channels and transporters are responsible for numerous human diseases, elucidation of their role in pathophysiology constitutes the first step in improving clinical management and developing therapeutic interventions. The ABC transporter superfamily of proteins has been implicated in a wide range of human diseases (Table 1),

such as CF and PHHI. Consequently, determination of the structure-function relationship of ABC transporters with regard to their intrinsic channel/transporter activity as well as their regulation of heterologous channels may provide insights into disease process which can be translated into alternative targets for therapy. Of particular interest is the ability of some ABC transporters to substitute for other members of the superfamily and perhaps to couple with multiple ion channels. Our initial studies suggest that this is due to the presence of specific motifs of ABC transporters, such as the NBFs. Mutations in the NBFs in CFTR and SUR1 have been associated with clinical CF and PHHI.

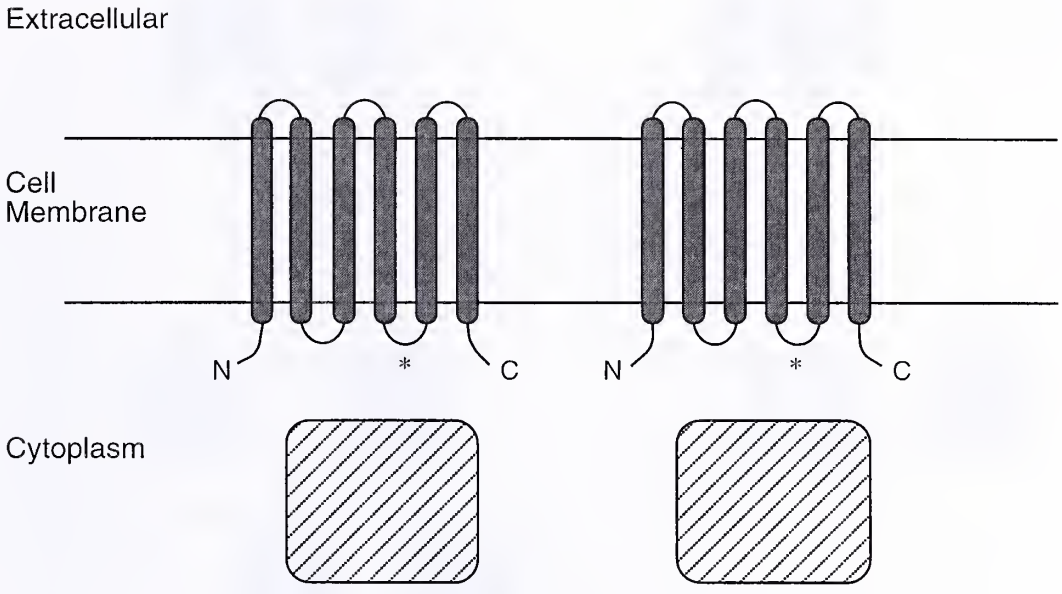


Figure 1. The Typical ABC Transporter. This is a schematic representation of a typical ABC transporter with its four core domains. The two nucleotide-binding domains are represented by the cross-hatched areas. The transmembrane domains are depicted here with six alpha-helical segments with the N- and C-termini on the cytoplasmic face. Although these domains are pictured as separate polypeptides here, they may also be fused to form a multidomain protein. The * designates the cytoplasmic loop which contains a short sequence motif conserved between many ABC transporters. Adapted from Higgins, 1992.

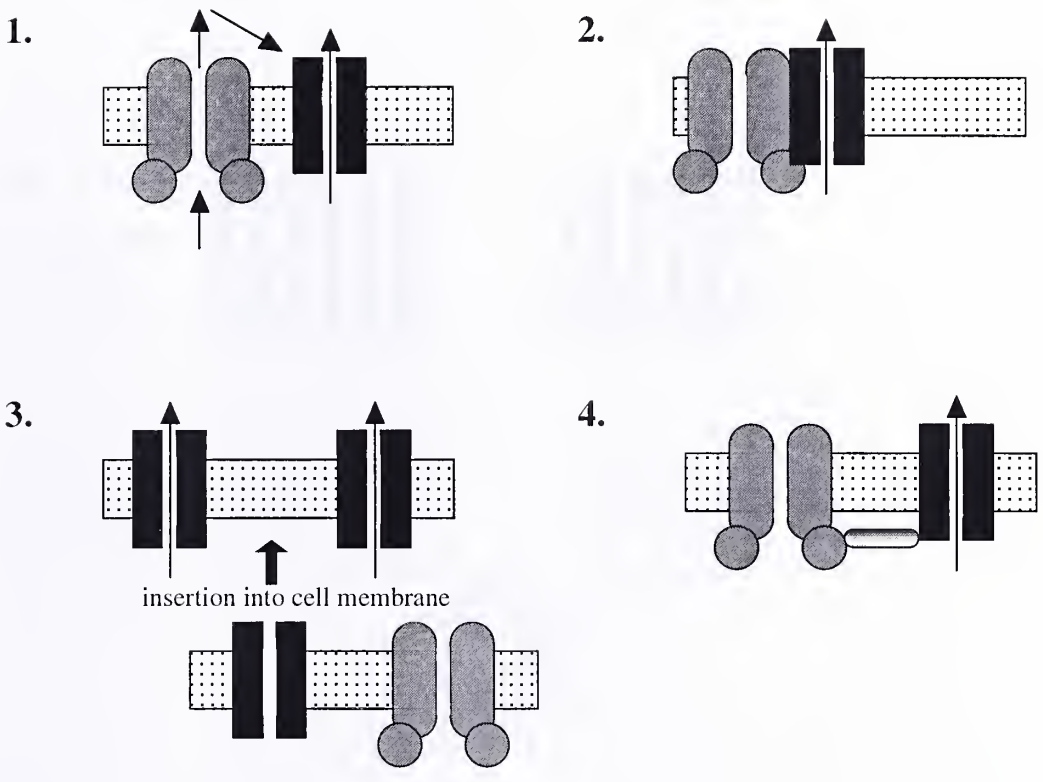


Figure 2. Mechanisms of Channel Regulation.
1. The substrate (X) transported by the ABC transporter acts to modulate channel activity in an autocrine fashion.
2. The ABC transporter acts directly upon the channel protein.
3. The ABC transporter facilitates increased insertion of the channel protein into the membrane.
4. The ABC transporter regulates the channel protein via an intermediate protein molecule.

Adapted from Higgins, 1995.

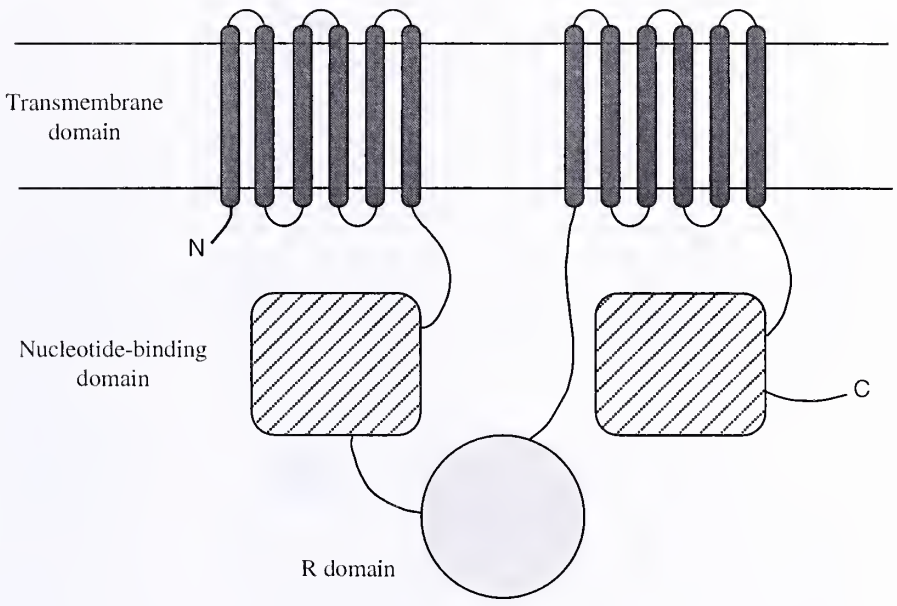


Figure 3. Model of the Cystic Fibrosis Transmembrane Conductance Regulator. This schematic illustration shows the atypical five domain structure of CFTR containing not only two transmembrane domains and two nucleotide-binding domains, but also as additional regulatory (R) domain.

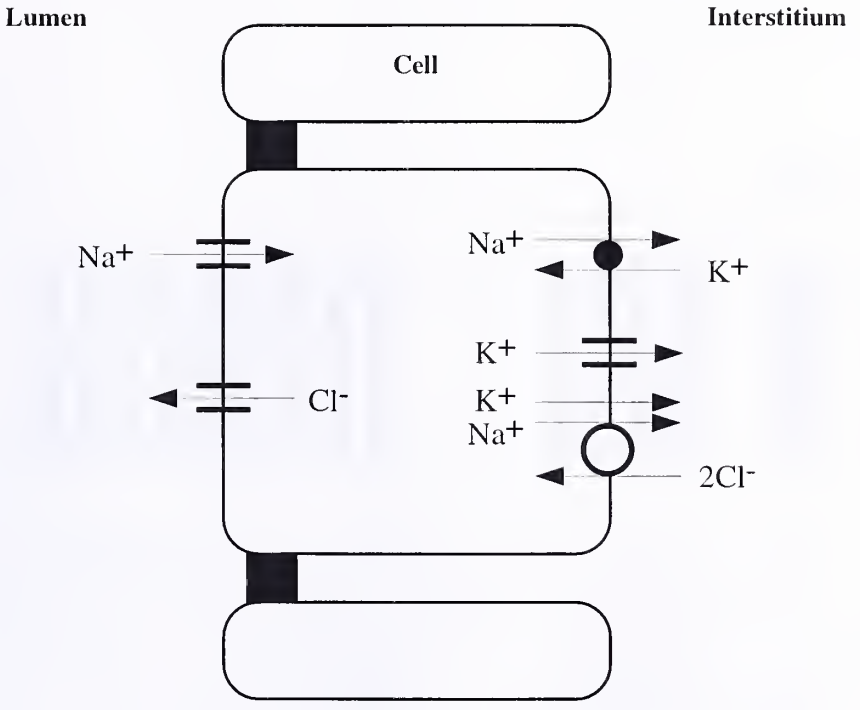


Figure 4. Ion Channels Involved in Regulation of Respiratory Airway Secretions. Chloride enters the cell across the basolateral membrane coupled to sodium movement out of the cell. This cotransport process may involve either a Na^+/Cl^- cotransporter or a $Na^+/K^+/2Cl^-$ cotransporter. The chloride then moves out of the cell through chloride channels on the apical membrane. This net movement of chloride is driven by the presence of the basolateral Na^+/K^+ pump, the basolateral potassium channel, and the apical sodium channel.

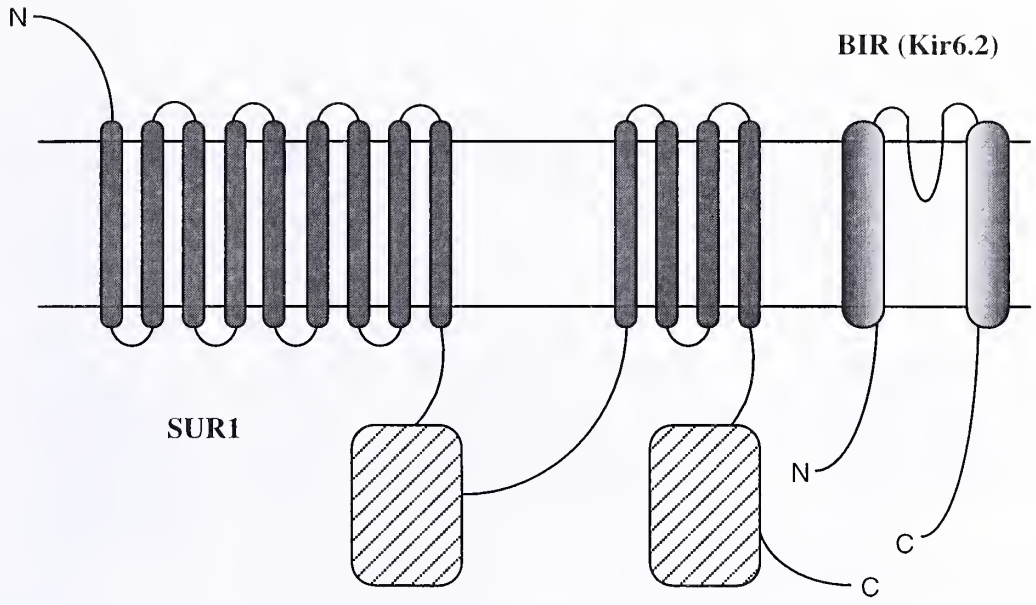


Figure 5. Proposed Model of the Pancreatic Beta Cell K_{ATP} Channel. The pancreatic beta cell K_{ATP} channel is postulated to be a complex of SUR1 and BIR (Kir6.2). Although we represent this relationship as one to one, the actual stoichiometry of this complex is unknown.

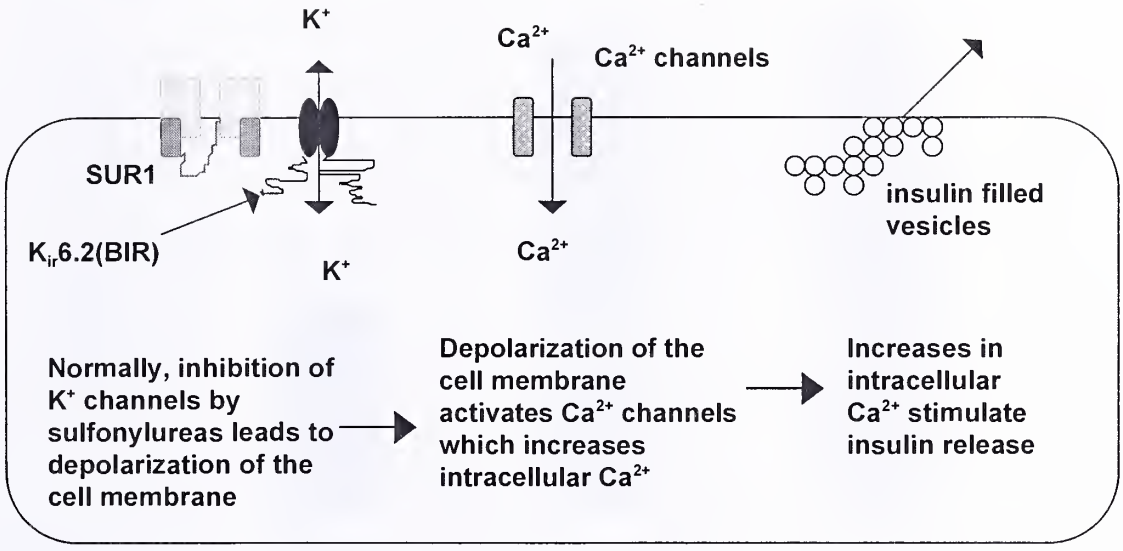


Figure 6. Glucose Homeostasis and Insulin Secretion. This figure illustrates the pathway for insulin secretion in the pancreatic beta cell. The pancreatic K_{ATP} channel (represented here as the SUR1 and BIR/Kir6.2 complex) maintains the resting membrane potential for the cell. An increase in the glucose concentration results in closure of the K_{ATP} channel. Depolarization of the cell activates the voltage-dependent calcium channels. The influx of calcium ions triggers exocytosis and insulin release. Sulfonylureas function by blocking the K_{ATP} channel and causing depolarization of the cell membrane.

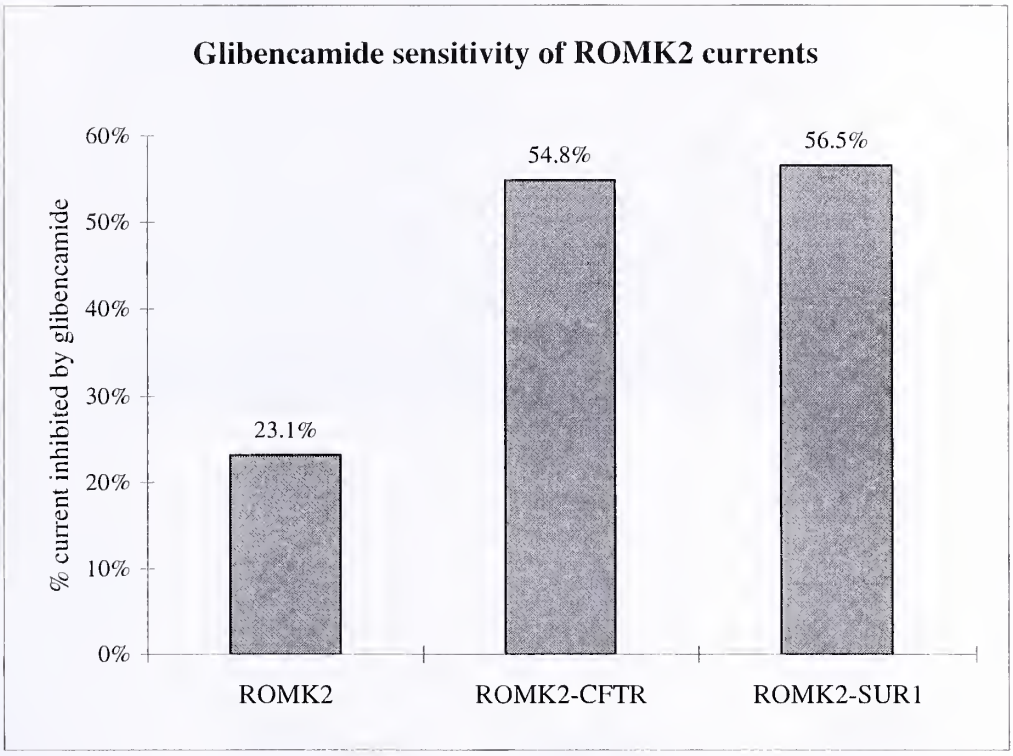


Figure 7. Glibenclamide Sensitivity of ROMK2 Currents. This figure is a summary of the data obtained for ROMK2 expressed alone, or with CFTR or SUR1 as indicated on the X-axis.

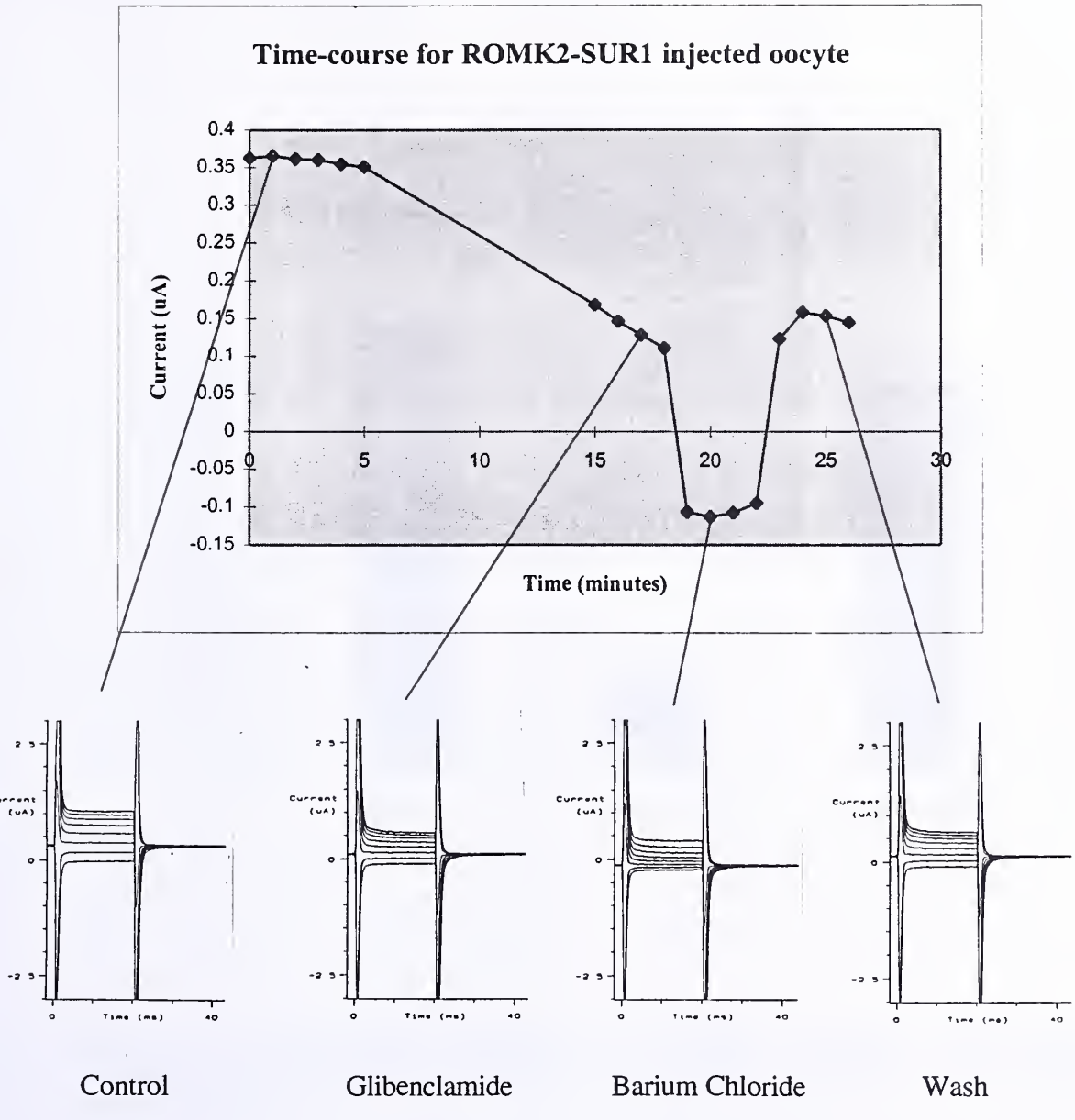


Figure 8. Time-Course and Individual Current-Voltage Traces. This is a representative time-course for an experiment in oocytes injected with ROMK2-SUR1. The individual current-voltage traces were taken at various points throughout the experiment. The protocol was as follows: 5 minutes control → 13 minutes in glibenclamide → 3 minutes in barium chloride → 5 minutes wash.

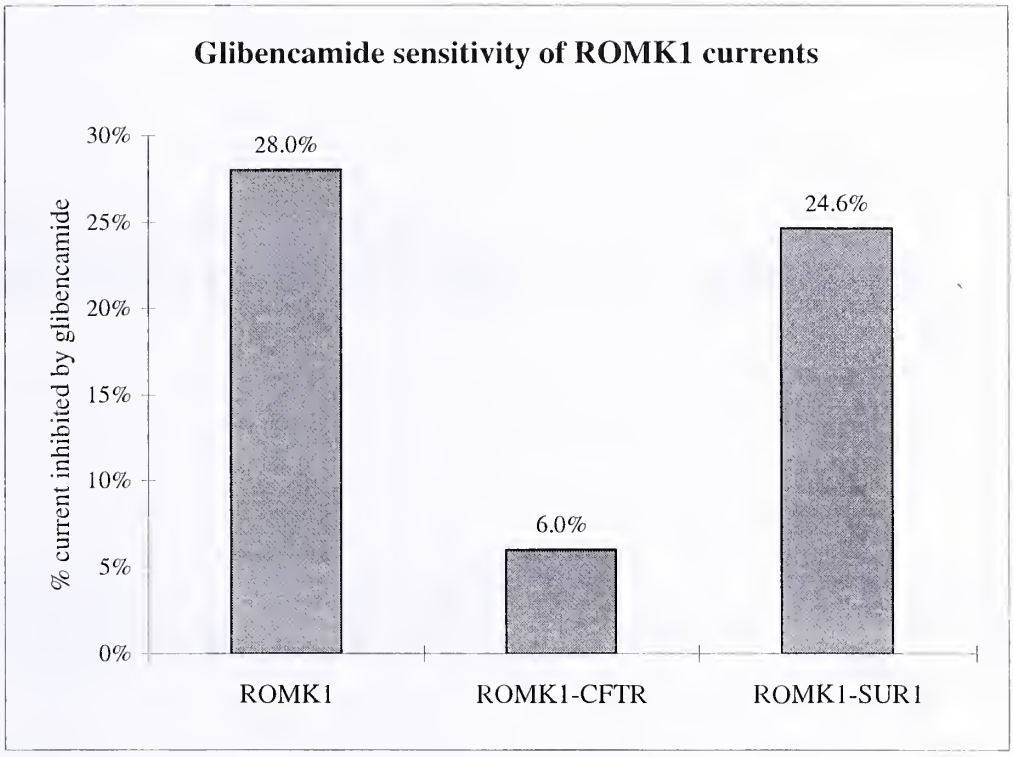


Figure 9. Glibenclamide Sensitivity of ROMK1 Currents. This figure is a summary of the data obtained for ROMK1 expressed alone, or with CFTR or SUR1 as indicated on the X-axis.

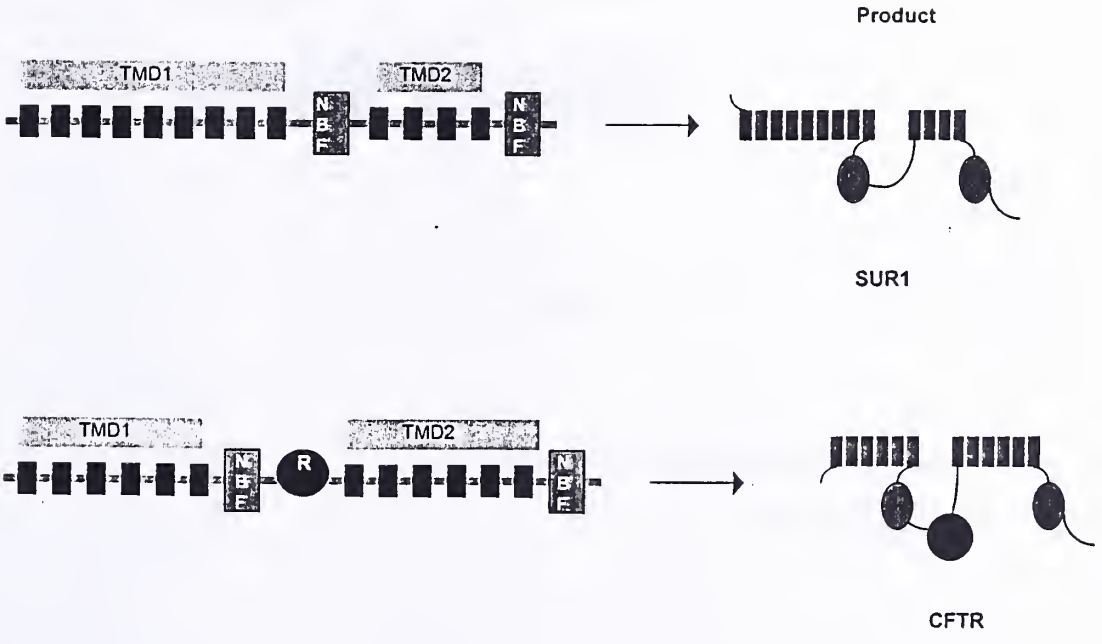
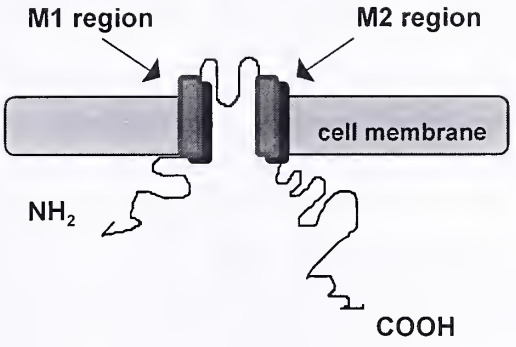


Figure 10. Comparison of Structure of CFTR and SUR1. Although CFTR and SUR1 are both members of the ABC transporter superfamily, each is a somewhat atypical example. Of note, CFTR has an additional regulatory (R) domain. SUR1 has a nine alpha-helical N-terminus transmembrane domain and a four alpha-helical C-terminus transmembrane domain. In addition, SUR1's N-terminus is extracellular while most ABC transporters, including CFTR, have a cytoplasmic N-terminus.



N-termini	
ROMK1	MGASERSVFRVLIRALTERMFKHLRRWFIT
ROMK2	MFKHLRRWFIT

Figure 11. Isoforms of the ROMK Renal K_{ATP} Channel. This figure illustrates the general structure of the K_{ir} family of potassium channels to which the ROMK channel belongs. ROMK1 (Kir1.1a) and ROMK2 (Kir1.1b) are two isoforms that differ in their N-terminus by 19 amino acids but are otherwise identical. The amino acid sequence of their N-termini are aligned above.

ABC Transporter	Association with Human Disease
ABCR	Stargardt macular dystrophy[1]
ALD	adrenoleukodystrophy[2]
CDR1	drug resistant candidosis[3]
CFTR	cystic fibrosis[3,4]
EhPgp1	drug resistant amoebiasis[3]
IdMDR	drug resistant visceral leishmaniasis[3]
pfMDR2	drug resistant malaria[3]
P-glycoprotein (MDR1)	multidrug resistance (cancer chemotherapy)[5]
PMP70	Zellweger syndrome[6]
SMDR2	drug resistant schistosomiasis[3]
SUR1	persistent hyperinsulinemic hypoglycemia of infancy[7,8], non-insulin dependent diabetes mellitus[9]
TAP1	Behcet's disease[10], multiple sclerosis[11]
TAP2	bare lymphocyte syndrome type 1[3], multiple sclerosis[11]
VgA	drug resistant wound infections, pneumonia, impetigo[3]

Table 1. ABC Transporters and Associated Diseases. These are some examples of human diseases associated with ABC transporters.

[1]Allikmets et al., 1997; [2]Mosser et al., 1993; [3]Decottignies and Goffeau, 1997; [4]Riordan et al., 1989; [5]Gill et al., 1992; [6]Gartner et al., 1992; [7]Bryan and Aguilar-Bryan, 1996; [8]Thomas et al., 1995; [9]Inoue et al., 1996; [10]Gonzalez-Escribano et al, 1995; [11]Middleton et al, 1994.

Class of Defect	Examples	Domain
I. Protein production	G542X	NBD1
	3905 insT	NBD2
	621 + G→T	TMD1
II. Protein processing/trafficking	ΔF508	NBD1
	ΔI507	NBD1
	S549I	NBD1
	S549R	NBD1
	A559T	NBD1
	N1303K	NBD2
III. Channel regulation/activation	G551D	NBD1
	G551S	NBD1
	G1224E	NBD2
	S1255P	NBD2
	G1349D	NBD2
IV. Chloride conductance	R117H	TMD1
	R334W	TMD1
	R347P	TMD1

Table 2. Classes of CFTR Mutations That Result in Cystic Fibrosis. CFTR mutations can be separated into four classes of defects. Some examples of these mutations and what domain they effect are listed above. NBD=nucleotide-binding domain, TMD=transmembrane domain. Adapted from Welsh, et al., 1993.

Summary of Data in ROMK Injected Oocytes

ROMK2 Injected Oocytes

Construct	% Inhibition	p=	n=
ROMK2	23.1±6.1	—	11
ROMK2-CFTR	54.8±10.8	0.02	11
ROMK2-SUR1	56.5±6.7	0.001	13

ROMK1 Injected Oocytes

Construct	% Inhibition	p=	n=
ROMK1	28±2.8	—	8
ROMK1-CFTR	6±0.02	0.00006	6
ROMK1-SUR1	14.6±6.3	0.6	6

Table 3. Summary of Data in ROMK Injected Oocytes. The percent inhibition is expressed as mean±SE. P values are based upon Student t-test. Comparison was made to ROMK2 or ROMK1 alone, as appropriate.

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